Ecotoxicology and Environmental Safety 81 (2012) 122–126

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Combined toxicity of cadmium and lead on the earthworm *Eisenia fetida* (Annelida, Oligochaeta)

Bing Wu [a], Zhengtao Liu [b], Yun Xu [a], Dingsheng Li [a], Mei Li [a,*]

[a] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, 210046, PR China
[b] State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environment Sciences, Beijing 100012, PR China

ARTICLE INFO

Article history:
Received 28 March 2012
Received in revised form
1 May 2012
Accepted 4 May 2012
Available online 16 May 2012

Keywords:
Cadmium
Lead
Earthworm
Combined toxicity
Comet assay

ABSTRACT

Cadmium (Cd) and lead (Pb) in soil have received extensive attention due to their potential toxicological effects. This study analyzed the combined toxicity of Cd and Pb on the earthworm *Eisenia fetida*. Cellulase activity and DNA damage were chosen as toxic endpoints. Factorial analysis was applied to identify the interaction of Cd and Pb. The results showed that single Pb and Cd could increase the cellulase activity and DNA damage of coelomocytes. The combination of both metals could significantly inhibit cellulase activity. For low Cd concentration, the addition of Pb could increase the DNA damage. However, for high Cd concentration, Pb could decrease the DNA damage. Factorial analysis showed that the changes of Cd concentrations exerted the highest influence on the combined toxicity, followed by factor "Cd*Pb" and "Pb". The combined toxicological effects between Cd and Pb were complex, which might be influenced by the competition adsorption of both metals in soil and biomembrane and their bioavailability. The results of this study are useful for understanding of combined toxicity of Cd and Pb on terrestrial invertebrates.

1. Introduction

Heavy metals are continuously being added to soils through various agricultural and industrial activities, which pose potential threat on food safety and health risks. Among heavy metals, cadmium (Cd) and lead (Pb) in the soil are receiving extensive attention in China, since Cd and Pb exert acute and chronic toxicological effects on plants and animals (Cheng, 2003; Li et al., 2009; Wei and Yang, 2010). When Cd and Pb coexist in the soil, their combined effects have proved to be very complex. There were many literatures concerned with combined effects of Cd and Pb on plants and phytoplankton (An et al., 2004; Fargasova, 1999; Manzo et al., 2010; Xu et al., 2009). However, very little is known about combined toxicological effects of Cd and Pb on terrestrial invertebrate. Among terrestrial invertebrates, earthworms are commonly adopted as target species because they are widely available, easily reared in laboratory and reproduce rapidly and steadily(Gomez-Eyles et al., 2009). Several organizations have developed earthworm protocols to assess the effects of chemicals on terrestrial invertebrates, such as ISO, EPA and OECD (Hirano and Tamae, 2011; Nahmani et al., 2007). Although these tests have been widely applied to evaluate toxicological effects on earthworms, many sub-lethal effects such as changes in behavior, reproduction, inhibition of enzyme activities and DNA damage are not addressed in standard acute toxicity tests.

The activities of enzymes in earthworms have been regarded as fast and prognostic indices of environmental stress. Cellulase is one of the most commonly studied digestive enzymes in earthworm, since its activity could directly influence the earthworm's ability to decompose plant litter and other cellulosic materials (Hu et al., 2010; Tejada et al., 2010). Cellulase activity has been used as a bio-indicator for pollutants in soil. DNA damage might lead to mutations, strand breaks and eventually carcinogenesis or teratogenesis. Therefore, DNA damage has been regarded as an important endpoint in toxicity testing. Among numerous assays used to detect DNA damage, the comet assay possess various advantages such as its sensitivity for detecting low levels of DNA damage in single cells and the relative ease of application (Collins, 2004; Tice et al., 2000). Comet assay has been widely used in ecotoxicology (Erbe et al., 2011; Mamaca et al., 2005; Park and Choi, 2007).

In this paper, we determined the combined toxicity of Cd and Pb on cellulase activity of earthworm (*Eisenia fetida*), and applied the comet assay to assess the DNA damage in coelomocytes of earthworms. Our objective is to improve our understanding on the combined effects of Cd and Pb on earthworms and provide more information on the combined ecological risk of both metals in soil ecosystems.

* Corresponding author.
E-mail addresses: pandanju@gmail.com, meili@nju.edu.cn (M. Li).

0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2012.05.003

2. Materials and methods

2.1. Test organism and exposure experiment

E. fetida were purchased from a farming factory in Jurong, Jiangsu Province, China. Healthy earthworms of about 60 days old, with 200–300 mg and a well-developed clitellum, were used for exposure experiments. Artificial soil of pH 6.0 ± 0.5, containing 10% finely ground sphagnum peat, 20% kaolin clay, 70% industrial sand was applied to raise earthworms.

Cadmium chloride ($CdCl_2$) and lead nitrate ($Pb(NO_3)_2$) (> 99%) were obtained from Sinopharm Chemical Reagent Co., Ltd. and Beijing Yili Fine Chemicals Co., Ltd., respectively. Individual metal ion was dissolved in distilled water to form different concentrations. Cd and Pb were added into the soil by mixing solutions of different concentrations. Three final concentrations for Cd and Pb referred in our previous studies were applied, which were 0.1, 1 and 10 mg/kg dry soil for Cd and 5, 50 and 500 mg/kg dry soil for Pb.

Before exposure experiment, the earthworms were acclimated in non-spiked artificial soil for one week. Then, ten of *E. fetida* were applied to perform metal exposure for each group. The contaminated soil with different concentrations and earthworms was placed in 1 L wide-mouth bottles. The conditions of experimental process were as follows continuous light source, 20 ± 1 °C temperature, and exposure period of 7 days. Water was sprayed into the room of bottle regularly to keep 80% air moisture, and the bottles were covered with plastic film that had been punched with small holes.

2.2. Biochemical assay

Protein content and cellulase activity in earthworms exposed to different combined concentrations of Cd and Pb were determined. Five earthworms for each group were applied. Proteins were extracted following the method of Mishra and Dash (1980) with some modifications. Firstly, five earthworms were rinsed with distilled water and dried on filter paper. Then, they were homogenized for 2 min in cold distilled water using JY92-II homogenizer (Ningbo, China). After homogenates were centrifuged at 700*g* for 10 min, the supernatant fluid was centrifuged at 1000*g* for 5 min. The final supernatant fluid was collected to test protein content and cellulase activity by the method provided by our previous report (Li et al., 2009).

2.3. Comet assay

DNA damages of earthworm coelomocytes were determined using comet assay. Coelomocytes were obtained according to the non-invasive extrusion method (Eyambe et al., 1991). In detail, five earthworms were rinsed in the extrusion medium (pH 7.3), containing 5% ethanol, 95% saline, 2.5 mg/ml Na_2-EDTA and 10 mg/ml guaiacol glyceryl ether. Coelomocytes could be spontaneously secreted in the medium. The obtained coelomocytes were washed twice with phosphate-buffered saline (PBS) and collected by centrifugation at 1000*g* for 3 min. Before comet assay, the coelomocytes were kept at 4 °C.

The comet assay was performed according to the methods of Singh et al. (1988) and Tice et al. (2000) with some modifications. Firstly, coelomocytes were embedded in frosted microscope slides by an agarose sandwich. Then, the slides were submerged in cold lysis solution (2.5 M NaCl, 10 mM Tris, 1% N-Lauroyl Sarcosine Na, 100 mM EDTA) in the darkness for 1 h. Slides were placed on a horizontal electrophoresis unit. Electrophoresis was conducted at 20 °C using 25 V and 300 mA for 30 min. After electrophoresis, slides were washed three times with 0.5 M Tris buffer (pH 7.5), and the DNA was stained with ethidium bromide. The stained slides were examined with a fluorescent microscope (BX41, Olympus, Japan). Five slides per group were prepared and at least 50 cells were analyzed for each slide. Photos were taken with a digital camera (C-5050ZOOM, Olympus). Images were analyzed by the comet assay software (CASP 1.2.2) according to the method of Collins et al. (1995).

2.4. Statistical analysis

Differences between control and treated groups were analyzed by one-way ANOVA test followed by Tukey's post hoc test ($p < 0.05$). The normality of data was checked by Kolmogorov–Smirnov test. Factorial analysis was performed to identify the interaction of Cd and Pb. All the statistical analysis was carried out in Minitab 14.0 software.

3. Results

3.1. Biochemical responses of combined Cd and Pb in E. foetida

The changes of protein content and cellulase activities in *E. foetida* exposed to combination of different Cd and Pb concentrations were expressed in terms of the ratio of measured data in experimental group and control group, which was shown in Table 1. As shown in the table, the protein contents were significantly increased or decreased by combination of high Cd and Pb concentration ($p < 0.05$). However, no obvious regulations of combined toxicological effects were identified. For cellulase activity, single Pb and

Table 1
Changes of protein content and cellulase activity in *E. foetida* exposed to combined Cd and Pb.

Cd (mg/kg dry soil)	Pb (mg/kg dry soil)	Protein content[a]	Cellulase activity[a]
0	5	0.93 ± 0.004^b	1.31 ± 0.008^b
0	50	0.98 ± 0.005	1.13 ± 0.017^b
0	500	0.97 ± 0.012	1.23 ± 0.017^b
0.1	0	1.10 ± 0.004^b	1.15 ± 0.015^b
0.1	5	0.864 ± 0.010	1.106 ± 0.016^b
0.1	50	1.077 ± 0.003^b	0.880 ± 0.016^b
0.1	500	0.819 ± 0.003^b	1.057 ± 0.013
1	0	1.11 ± 0.004^b	1.04 ± 0.015
1	5	0.974 ± 0.006	0.967 ± 0.010
1	50	1.080 ± 0.007^b	0.887 ± 0.010^b
1	500	1.094 ± 0.002^b	0.803 ± 0.011^b
10	0	0.93 ± 0.003^b	1.09 ± 0.015
10	5	1.019 ± 0.008	1.008 ± 0.009
10	50	1.092 ± 0.008^b	0.858 ± 0.017^b
10	500	1.056 ± 0.005^b	0.732 ± 0.003^b

[a] Protein contents and cellulase activity were shown in terms of ratio of measured data in experimental groups and control group (Mean ± S.E.).
[b] Significant difference compared with the control (ANOVA) $p < 0.05$.

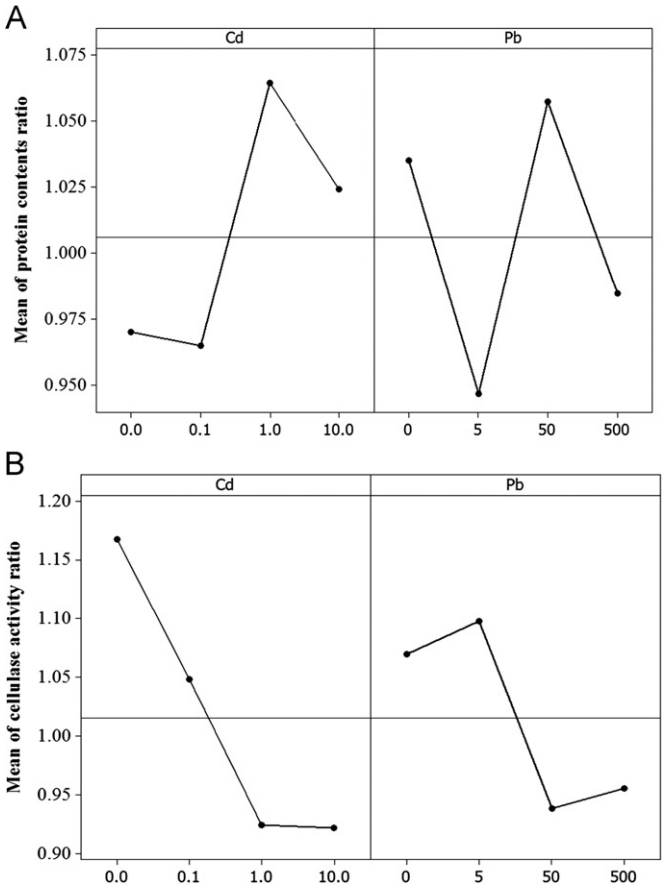

Fig. 1. Combined influences of different Cd and Pb concentrations on the protein content (A) and cellulase activity (B) under combined exposure obtained by factorial analysis. The horizontal coordinate represents the concentrations of Cd and Pb. Their unit is mg/kg dry soil.